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Award Number: W81XWH-10-1-0432

TITLE: Biology and Therapeutic Relevance of a Novel Kinase Pathway in Refractory Breast Cancers

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REPORT DATE: July 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 1 Jul 2011		2. REPORT TYPE Annual Summary		3. DATES COVERED 15 JUN 2010 - 14 JUN 2011	
4. TITLE AND SUBTITLE Biology and Therapeutic Relevance of a Novel Kinase Pathway in Refractory Breast Cancers				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0432	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Lei He, Ph.D. E-Mail: lhe1@partners.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Massachusetts General Hospital Boston, MA 02114				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT A particularly treatment-refractory subtype of breast cancer is the so-called "triple negative" breast cancer (TNBC). Our laboratory has recently identified a novel kinase, Ser/Thr kinase Stk36, which can regulate p63/p73 pathway in TNBC. Stk36 is overexpressed, genomically amplified, and subject to missense mutations in cancers including breast cancer. I therefore hypothesize that Stk36 is an endogenous apoptosis suppressor in TNBC whose overexpression and/or mutation may confer chemo-resistance. During the 1st funding year, I demonstrated that the apoptosis induced by Stk36 inhibition can be completely rescued by p73 ablation. P63 level and Hedgehog pathway activity are not involved in this process; p73 protein may translocate into more tightly chromatin-bound state (thus more transcriptionally active). In addition, overexpression of wildtype Stk36 confers modest resistance to cisplatin treatment in vitro.					
15. SUBJECT TERMS TNBC, Stk36, p73, endogenous inhibitor of apoptosis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER (include area code)

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Introduction

A particularly treatment-refractory subtype of breast cancer is the so-called “triple negative” breast cancer (**TNBC**), which lacks estrogen and progesterone receptor expression and Her-2 amplification. To date little progress has been made in identifying specific molecular pathways of therapeutic value for TNBC. Our laboratory has recently identified a chemosensitivity pathway in these tumors which involves the p53-related transcription factors p63 and p73. A genome-wide RNA interference screen was carried out to identify regulators of this pathway that might represent future therapeutic targets for TNBC. The most novel and potentially important regulator gene uncovered in this screen is the Ser/Thr kinase Stk36, which is overexpressed, genomically amplified, and subject to missense mutations in cancers including breast cancer. I therefore hypothesize that Stk36 is an endogenous apoptosis suppressor in TNBC whose overexpression and/or mutation may confer chemoresistance. My objective is to uncover the biochemical pathway for Stk36 function and to determine its clinical relevance in TNBC.

Body

Task I.1. *Stk36 interaction with p63/p73 pathway:*

Subtasks:

a) data about whether p73 knockdown can reverse the apoptosis induced by Stk36 ablation;

In the preliminary data section, I used (Dharmacon) siRNA transfection method to achieve transient Stk36 inhibition. To corroborate the previous experimental result with an independent approach, I decided to first validate the previous finding with retrovirus mediated shRNA knockdown approach. In the protocol developed in our lab, retrovirus mediated shRNA knockdown can lead to potent and long-term inhibition of gene expression; this method is also necessary for the biochemical work I proposed in my **SOW II**, because it can bring down the cost of Stk36 inhibition in large scale experiment. As a member of MGH cancer center, I have access to Broad Institute RNAi consortium, which has a collection of 10 premade shRNA clones against human Stk36 gene. With retroviral infection on MDA-MB-468 triple negative breast cancer cells, I tested the knockdown effect of these 10 clones against human Stk36 transcript, and identified two most

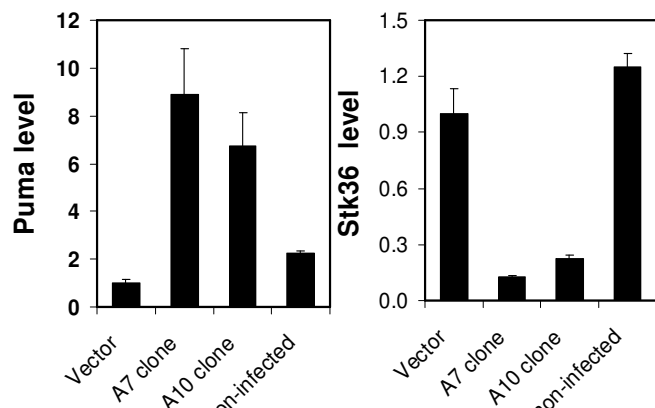


Fig.1. Knockdown Stk36 with shRNA leads to potent Puma activation.

effective shRNA clones, namely A7 and A10. These two shRNA clones can lead to potent Stk36 inhibition at mRNA level (Fig.1.) and protein level (data not shown), as well as potent p73 target gene induction (see left figure).

As a second step to see if the status of p73 has any effect on the cellular response to acute Stk36 ablation, I did sequential knockdown experiment on MDA-MB-468 cell line. First, I knocked down p73 with retrovirus packaging shRNA against p73; 4 days later following the 1st round of infection, I infected cells again with retrovirus packaging shRNA against Stk36. Three days post 2nd round of infection, I assayed for cell death and p73 target gene induction.

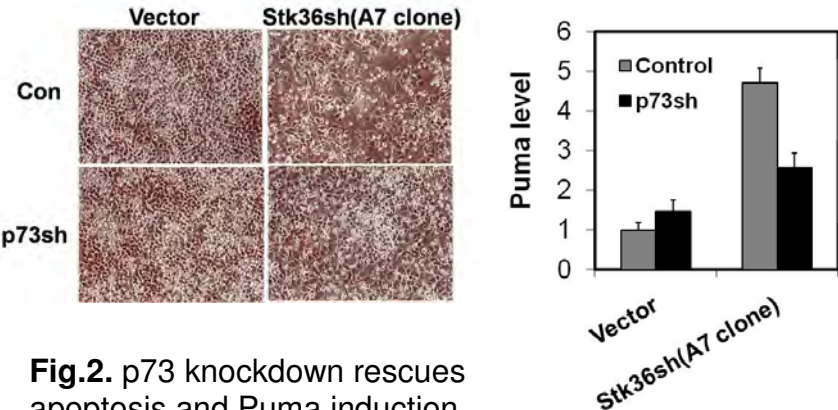


Fig.2. p73 knockdown rescues apoptosis and Puma induction induced by Stk36 ablation.

In cells with intact functional p73, Stk36 inhibition leads to p73 target gene Puma induction and apoptosis (see left figure). In cells where p73 was knocked down with shRNA, cells were completely resistant to apoptosis stemming from Stk36 inhibition (Fig.2), and Puma transcript was not up-regulated (Fig.2). This experiment clearly demonstrated that p73 knockdown can reverse the apoptosis response

induced by stk36 ablation.

b) correlation data of p73 status and Stk36 induced apoptosis in cancer line lines:

From the most recent mutation data in the COSMIC database of Sanger Center (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>), it appears that Stk36 mutation occurs more often in ovarian cancer patients than in any other types of cancer patients. I therefore reasoned that Stk36 may also be playing indispensable roles as an endogenous inhibitor of p73 induced apoptosis in ovarian cancers. This hypothesis fits well with my previous

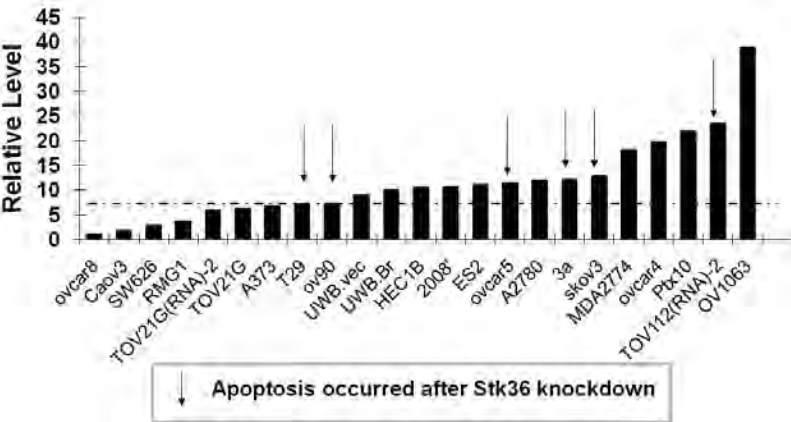


Fig.3. Stk36 expression in human cancer cell lines.

characteristics of p73 dependent apoptotic response to cisplatin based therapy. From a collection of human ovarian cancer cell lines, I

first surveyed Stk36 mRNA level by QRT-PCR (Fig.3). Second, I chose several cell lines with various degrees of Stk36 expression levels and infected them with retrovirus packaging shRNA against Stk36. Not surprisingly, all ovarian cancer cell lines tested showed apoptosis upon Stk36 knockdown. In the figure I presented here, the cells in which I have tested for Stk36 dependency were marked with an arrow.

c) Data about the biochemical changes of p63, p73 and their downstream genes following Stk36 knockdown.

The up-regulation of p73 downstream genes including Puma and Noxa could be due to either p63 inhibition of p73 activation (2, 3). To test the two possibilities, I performed IP-Western of p63 and p73 following Stk36 knockdown in MDA-MB-468 cells. The result shows that p63 protein remained unchanged following Stk36 knockdown (see left figure), indicating the up-regulation of Puma is not due to decrease of p63 mediated inhibition. Furthermore, I also did reciprocal co-IP of p63 and p73, which clearly showed that there is no change between the associations of these two proteins (data not shown). I therefore hypothesize that Puma and Noxa up-regulation result from p73 activation. Intriguingly, p73 level decreased following Stk36 knockdown (Fig.4). This may be paradoxical at first glance; however, it can also indicate that p73 translocates from soluble nuclear fraction into tightly chromatin bound fraction (which cannot be immuno-precipitated), thus leading to the activation of p73 downstream genes, including Puma and Noxa. I am currently testing this hypothesis under more stringent stronger lysis condition.

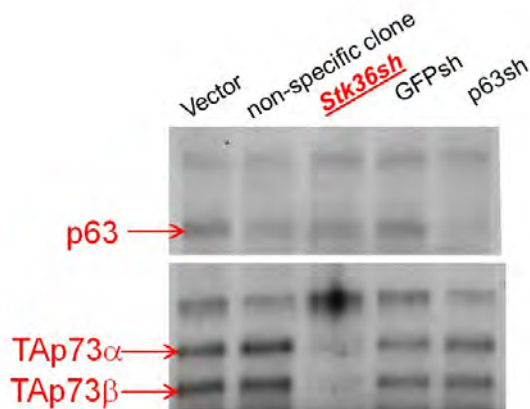


Fig.4. p63 and p73 IP-Western following Stk36 knockdown.

Task I.2. Involvement of Hedgehog pathway:

Subtasks:

a) data about Hedgehog status after Stk36 knockdown:

I knocked down Stk36 with retrovirus packaged shRNA, and then assayed mRNA level for Stk36, Puma, Noxa and Ptch1 on day 3 and day 4 post retroviral infection. Stk36 inhibition, Puma and Noxa up-regulation occurred on Day 3 and peaked on Day 4; significant cell death began being observed on Day 4. However, under the time line where I observed Stk36 inhibition, Puma/Noxa up-regulation, and cell death, I didn't see significant change in Hedgehog pathway alteration as assessed by its target gene level Ptch1 mRNA (Fig.5).

Although Stk36 or its homolog gene Fused is an

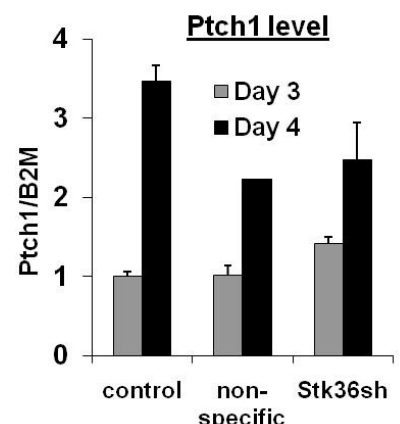


Fig.5. Kinetic change of Ptch1 following Stk36 knockdown.

indispensable positive regulator for Hedgehog pathway in *Drosophila*, the role of Stk36 in mammalian Hedgehog pathway is controversial and conflicting. There is no obvious Hedgehog pathway alteration in Stk36 knockout mice. My conclusion is in triple negative cancer cells whose survival depends on Stk36, Hedgehog pathway is not disrupted following Stk36 ablation, and therefore is not involved in the apoptosis response.

b) data about whether high-level Stk36 overexpression can block apoptosis induced by cyclopamine (**not applicable**):

c) data about whether overexpression of Gli can block cell death induced by Stk36 knockdown (**not applicable**):

Task I.3. Domain and mutant characterization of Stk36:

Subtasks:

a) Molecular cloning of RNAi resistant Stk36 domain mutant and tumor associated mutants:

First I cloned the open reading frame (**ORF**) of Stk36 gene from a cDNA pool generated from a normal human foreskin sample. The size of Stk36 ORF is 3.9 kb, and the cloning procedure turned out to be non-trivial. The cloned fragment was then transferred to a retroviral vector pMSCV. In this vector, Stk36 is under the control of CMV promoter to ensure expression in mammalian cells. I made two pMSCV constructs, one construct contains untagged Stk36; the other construct contains Stk36 tagged with Ha and Flag in a tandem way at the C terminus. Next, I stably transduced Stk36 and tagged Stk36 into NIH3T3 mouse fibroblast cells, MDA-MB-468 human triple negative breast cancer cells, and JHU-029 human cancer cells (in this cell line, TAp73 β open reading frame was put under an Tet inducible promoter) (3). These cell lines were used for the analysis listed in subtask **I.3c**. In the future, the tagged Stk36 construct will be used for the proposed tandem IP/Mass-Spec experiment in my **SOW II** (to identify Stk36 associated proteins or substrates).

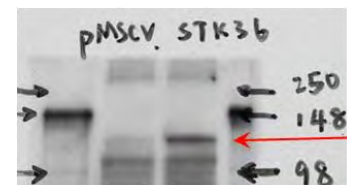


Fig.6. Stk36 Western in control and over-expressing cells.

b) Rescue data by ectopic expression of these mutants following endogenous Stk36 knockdown:

shRNA resistant Stk36 wild type construct and tumor associated mutant constructs were generated with Stratagene Mutagenesis kit. I am currently making MDA-MB-468 cell lines stably expressing these constructs. Rescue experiment will be promptly done after all the cells being generated.

c) Cisplatin sensitivity data in reconstituted cell lines:

I first tested the cisplatin sensitivity or resistance status in JHU-029 cells that I had generated by over-expressing wild type Stk36. This particular JHU-029 cell line can express TAp73 β in the presence of low concentration of tetracycline, and thus leading to decreased cell viability (Fig.7). I tested cisplatin sensitivity in the absence of tetracycline, as well as in the presence of 1 μ g/ml tetracycline to induce high levels of p73. Results (Fig.7) show that under pulsed cisplatin treatment condition (2 hr treatment, followed by washing and re-feeding with fresh medium), Stk36 over-expressing cells have a higher viability (though modest) than the control cells transduced with empty vector. This difference exist both at baseline p73 status (without tetracycline induction), or high p73 expression condition (induced by tetracycline). This result indicates that over-expression of Stk36 can inhibit the pro-apoptotic function of endogenous p73, as well as extraneously expressed p73 protein. This line of observation is consistent with the previous Stk36 knockdown study, where ablation of Stk36 induced the pro-apoptotic function of endogenous p73 protein.

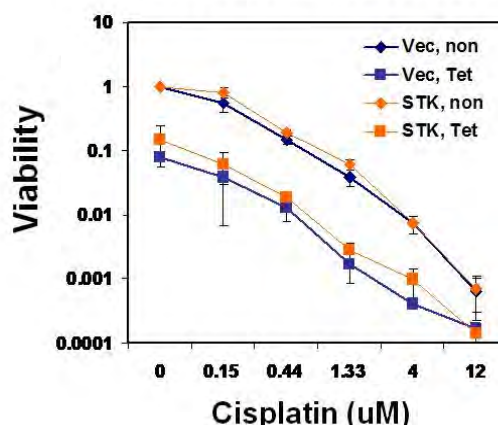


Fig.7. Cisplatin dose response curve.

Task I.4. identify small molecule inhibitors for Stk36 kinase (*not listed in the original SOW*).

My mentor has established collaboration with Dr. Nathanael Gray, a highly-experienced academic medicinal chemist whose work focuses on kinase inhibition as a therapeutic strategy. Dr. Gray has in hand several small molecule kinase inhibitors which exhibited high affinity binding to Stk36 in their assay. I was fortunate to obtain these chemical compounds. My initial approach will be to use our p73-dependent reporter lines in order to screen these inhibitors for their ability to activate p73-dependent transcription and death. Confirmation of the target specificity will then be performed by rescue experiments to test whether overexpression of the Stk36 kinase (versus kinase-dead control) can abrogate the effect of the chemical inhibitor.

Key Research Accomplishments

- Identified two effective shRNA clones that lead to potent Stk36 inhibition;
- Acute Stk36 inhibition leads to Puma/Noxa upregulation and apoptosis; both are p73-dependent;
- No change in p63 level and the association between p63 and p73 following Stk36 ablation;
- No obvious change in Hedgehog pathway activity;
- p73 protein may translocate from nuclear soluble fraction into tightly chromatin-bound fraction and therefore lead to downstream gene activation;
- Stk36 over-expression leads to resistance to cisplatin;

- Generate Stk36 construct tagged with Ha and Flag (to be used in **SOW II**).

Reportable Outcomes

- Abstract and poster to be presented in Era of Hope meeting, Orlando Florida, August 2-5, 2011
- Development of cell lines
 - NIH3T3 cells stably transduced with Stk36 or Stk36.ha.flag;
 - MDA-MB-468 cells stably transduced with Stk36 or Stk36.ha.flag;
 - JHU-029 cells stably transduced with Stk36 or Stk36.ha.flag.

Conclusion

Stk36 kinase is an endogenous inhibitor for the pro-apoptotic activity of p73 in TN breast cancer patients. Targeting Stk36 with small molecule inhibitors is a promising approach to treat refractory TN cancers.

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Appendices - NONE